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TITLE: Targeting Nuclear Receptors to Treat Fibrostenotic Crohn's Disease

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14. ABSTRACT While current therapies are effective in many patients with Crohn's disease (CD), others exhibit complications that require surgery. Fibrosis and increased smooth muscle (SM) thickening contributing to stricture formation and intestinal obstruction, occurs in 30-50% of CD patients within 10 years of disease onset. NR4A1 is an orphan nuclear receptor that has recently been identified as a key regulator of fibrosis and cell growth in non-intestinal systems. We found that NR4A1 activation reduces fibrosis and SM thickening, caused by established chronic inflammation, in a spontaneous CD-like model of ileitis (SAMP/YitFcSJ mouse). Deletion of Nr4a1 enhances fibrosis and SM thickening in the chronic DSS-model of colitis. <i>In vitro</i> , exposing primary intestinal fibroblasts (IF) to TGF-β1 enhances the expression of NR4A1, whereas, NR4A1 activation suppressed TGF-β1-induced expression of fibrotic genes, supporting the existence of an NR4A1-TGF-β1 negative feedback loop.					
15. SUBJECT TERMS Crohn's disease, intestinal smooth muscle, intestinal fibroblasts, NR4A1, 6-MP, Cytosporone B, proliferation, inflammation					
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## **INTRODUCTION**

While current therapies are effective in many patients with Crohn's disease, others exhibit complications that require surgery. Fibrosis and increased muscle thickening contributing to stricture formation and intestinal obstruction, occurs in 30-50% of Crohn's disease patients within 10 years of disease onset. Unfortunately, more than 50% of those who undergo surgery will experience stricture recurrence. Despite the advances in the treatment of Crohn's disease, current therapies do little to prevent or reverse stricture formation. Thus, new targets must be identified to address this unmet health care need.

NR4A1 is an orphan nuclear receptor that has recently been identified as a key regulator of fibrosis and cell growth in non-intestinal systems. NR4A1 activation inhibits smooth muscle cell proliferation; blocks the switch to the synthetic phenotype; modulates transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) signaling in fibroblasts; and attenuates fibrosis in a variety of organs. Furthermore, reports from the past year suggest that NR4A1 can inhibit intestinal inflammation.

Data generated over the course of this reporting period reveal that NR4A1 plays a significant role in regulating pathogenic intestinal tissue remodelling by modulating fibrotic, proliferative and inflammatory signaling in mesenchymal cells.

**KEYWORDS**

Crohn's disease, intestinal smooth muscle, intestinal fibroblasts, NR4A1, 6-mercaptopurine, Cytosporone B, proliferation, inflammation

## **ACCOMPLISHMENTS**

### **Major Goal/Specific Aim 1 – To determine whether NR4A1/Nur77 modulates the function of intestinal mesenchymal cell populations**

*Major Task 1 (MT1) – Assess the function of NR4A1/Nur77 in intestinal fibroblasts*

*Major Task 2 (MT2) – Assess the function of NR4A1/Nur77 in intestinal smooth muscle cells*

#### **Work accomplished**

In MT1, we sought to assess the role that NR4A1/Nur77 plays in regulating intracellular signaling pathways that regulate intestinal fibroblasts, driving fibrogenesis, cell proliferation and inflammatory mediator release. First, we found that treating primary human intestinal fibroblasts with TGF- $\beta$  triggered the upregulation of NR4A1 in the short-term (over the course of 6 hrs of TGF- $\beta$  exposure; Figure 1). However, long-term treatment of these cells with TGF- $\beta$ , led to a loss of this induction (Figure 1). To assess the role of NR4A1 in regulating fibrogenesis, we stimulated primary mouse intestinal fibroblasts with TGF- $\beta$  for 16 hrs, and assessed the expression of *Col1a1* and *Col1a2*, genes associated with tissue remodelling and fibrosis in the context of intestinal inflammation. Treating these cells with TGF- $\beta$  increased the expression of both *Col1a1* and *Col1a2*, however, pretreating the cells with an NR4A1 agonist (cytosporone B – CytoB) blocked the induction of these pro-fibrotic genes (Figure. 2). In preliminary experiments, it appears that NR4A1 activation may elicit this inhibitory effect through attenuating SMAD signaling, as its activation reduced TGF- $\beta$ -induced SMAD3 phosphorylation in primary mouse intestinal fibroblasts (Figure 3). Taken together, these data suggest that NR4A1 may act in a negative feedback loop to mimic the fibrogenic effects of TGF- $\beta$ . Interestingly, cytosporone B treatment also attenuated the production of fibrosis-associated inflammatory mediators (eotaxin, IL-9, IL-3 and IL-17A) released from primary mouse intestinal fibroblasts exposed to cytomix (TNF- $\alpha$ , IL-1 $\beta$ , IFN $\gamma$ ; Figure 4). Lastly, in MT1, we found that activation of NR4A1 with cytosporone B, but not 6-mercaptopurine (a partial NR4A1 agonist), attenuated serum-induced proliferation of primary human intestinal fibroblasts (Figure 5).

In MT2, we sought to characterize the function of NR4A1/Nur77 in the regulation of intestinal smooth muscle proliferation, a key driver of bowel wall thickening and luminal narrowing in fibrostenotic Crohn's disease. First, using Nr4a1-GFP reporter mice, we found that animals recovering from an acute induction of intestinal inflammation (dextran sulphate sodium/DSS model of colitis), exhibited increased expression of Nr4a1-GFP, most prominent at 7 days post-DSS exposure (Figure 6). In our cell-based studies, NR4A1 activation with cytosporone B attenuated serum-induced proliferation in primary human intestinal smooth muscle cells (Figure 7). This anti-proliferative effect was also observed in cells exposed to IGF-1 (Figure 8) and PDGF-BB (Figure 9), two mitogens thought to contribute to the remodelling observed in fibrostenotic Crohn's disease patients. Taken together, these data suggest that NR4A1 can control mitogen-induced proliferation of intestinal smooth muscle cells and may regulate these responses following inflammatory insult.

### Training opportunities

Nothing to Report

### Dissemination of results

Nothing to Report

### Plan for next report period

We would intend to assess key aspects of these findings in additional primary cell cultures (intestinal fibroblast and smooth muscle) as per the studies approved in our human ethics protocol. To date, the primary cell work has been conducted on cells purchased from a commercial vendor.

Furthermore, we need to assess cell viability and the induction of apoptosis in primary smooth muscle cells and fibroblasts to determine whether NR4A1 activation alters cell survival.

**Major Goal/Specific Aim 2 – To assess the role that NR4A1/Nur77 plays in regulating inflammation-induced pro-fibrotic/proliferative signaling in models of experimental IBD.**

*Major Task 1 (MT1) – Assess the role that NR4A1/Nur77 plays in inflammation-induced tissue remodelling in the DSS model of experimental colitis*

*Major Task 2 (MT2) – Assess whether pharmacological activation of NR4A1/Nur77 alters the disease course of the SAMP1/YitFcsJ model of Crohn's-like ileitis and tissue remodelling*

### Work accomplished

In MT1, we used the DSS model of experimental colitis to evoke inflammation and assess recovery and the degree of tissue remodelling in wild-type (WT) versus Nr4a1<sup>-/-</sup> mice. We found that chronic intestinal inflammation triggered colonic fibrosis and smooth muscle thickening in WT mice, but this response was significantly greater in Nr4a1<sup>-/-</sup> mice (Figure 10). While this work was being performed, it was reported that Nr4a1<sup>-/-</sup> mice were more susceptible in the acute DSS model, thus we sought to confirm this in our hands, as the degree of initial injury could be the driver of the tissue remodelling observed later in our experiments. Indeed, acute DSS exposure led to an increased disease activity index (DAI) score and enhanced colonic shortening in Nr4a1<sup>-/-</sup> mice when compared to their WT counterparts (Figure 11). To assess whether NR4A1 activation could attenuate the response to acute DSS-induced colitis, WT and Nr4a1<sup>-/-</sup> were treated with cyclosporine B (CSN-B) or 6-mercaptopurine (6-MP) during DSS exposure. In an attempt to trigger the same degree of disease in WT and Nr4a1<sup>-/-</sup> mice, the latter was exposed to a lower concentration of DSS (WT - 3.5% DSS in drinking water; Nr4a1<sup>-/-</sup> - 2.5% DSS in drinking water). Interestingly, CSN-B and 6-MP reduced body weight loss, a component of DAI, in WT, but not Nr4a1<sup>-/-</sup> mice (Figure 12). While these data are preliminary, they suggest that targeting NR4A1 may reduce overt disease presentation following DSS exposure.

In the MT2, we used the Crohn's-like ileitis model (SAMP1/YitFcsJ mouse) to assess the clinical efficacy of the NR4A1 agonist cytosporone B, on the induction of intestinal fibrosis and muscle thickening. The SAMP1/YitFcsJ mouse exhibits spontaneous ileal inflammation starting at 10-12 weeks of age, and usually presents with markers of chronic inflammation and remodelling at 18-20 weeks of age. In this set of experiments, we began cytosporone B treatment at 10 weeks of age, to mimic a treatment scenario versus a prophylactic approach. In these mice, cytosporone B treatment significantly reduced small intestinal fibrosis and smooth muscle thickening (assessed at 18 weeks of age; Figure 13).

#### Training opportunities

Nothing to Report

#### Dissemination of results

Nothing to Report

#### Plan for next report period

For MT1, we will need to repeat the chronic colitis experiments in the Nr4a1<sup>-/-</sup> mice to determine whether the exaggerated tissue remodelling observed in our studies reflects a hyper-fibrotic response, versus a hyper-inflammatory phenotype. Furthermore, we will assess the efficacy of cytosporone B and 6-MP in these experiments, to determine whether they reduce inflammation and tissue remodelling via NR4A1 activation.

For MT2, we will assess the function of primary intestinal mesenchymal cells isolated from cytosporone B-treated SAMP1 mice to determine whether they exhibit changes in cell signaling that control fibrogenesis, proliferation, survival and inflammatory mediator release.

### **Major Goal/Specific Aim 3 – To determine whether fibrostenotic CD is associated with aberrant NR4A1/Nur77 expression/function**

*Major Task 1 (MT1) – Examining the expression of NR4A1/Nur77 in human ileal resections*

*Major Task 2 (MT2) – Assessing the functional effect of NR4A1/Nur77 on mesenchymal cells isolated from CD resection samples*

#### Work accomplished

At this time, we have nothing to report for MT1 and MT2 associated with this Major Goal/Specific Aim. While our ethics protocol has been approved since the start of this contract/grant, we have had difficulties recruiting patients in our study.

#### Training opportunities

Nothing to Report



Dissemination of results

Nothing to Report

Plan for next report period

We have started a dialogue with our hospital's Dept. of Surgery and intend to recruit a surgical fellow as part of our study team in order to enhance patient recruitment and tissue sample acquisition.

## **IMPACT**

### *Impact on the development of the principal discipline(s) of the project*

The data generated within this reporting period provide insight into how NR4A1 contributes to the regulation of tissue inflammation and remodeling in Crohn's disease. Should our studies continue to support our hypothesis, and be backed by additional work using human tissues, they will provide the impetus to target NR4A1 to attenuate, and possibly reverse, the pathogenic remodeling observed in fibrostenotic Crohn's disease.

### *Impact on other disciplines*

Nothing to Report

### *Impact on technology transfer*

Nothing to Report

### *Impact on society beyond science and technology*

Nothing to Report

## **CHANGES/PROBLEMS**

### *Changes in approach and reasons for change*

Nothing to Report

### *Actual or anticipated problems or delays and actions or plans to resolve them*

As described in the progress from “Major Goal/Specific Aim 3 – To determine whether fibrostenotic CD is associated with aberrant NR4A1/Nur77 expression/function”, we have struggled with patient recruitment, limiting the ability of our lab to perform experiments and characterize the role of NR4A1 in primary intestinal smooth muscle cells and fibroblasts, isolated from fibrostenotic Crohn’s disease patient resection samples. To attempt to resolve this, we intend to recruit a surgical fellow as part of our study team. This approach will enhance interactions between our study team, the Dept. of Surgery, and their patients, with the goal of recruiting more patients into our study.

### *Changes that had a significant impact on expenditures*

Nothing to Report

### *Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents*

Nothing to Report

### *Significant changes in use or care of human subjects*

Nothing to Report

### *Significant changes in use or care of vertebrate animals*

Nothing to Report

### *Significant changes in use of biohazards and/or select agents*

Nothing to Report

## **PRODUCTS**

### *Publications, conference papers, and presentations*

*Journal publications*

Nothing to Report

*Books or other non-periodical, one-time publications*

Nothing to Report

*Other publications, conference papers, and presentations*

Nothing to Report

### *Website(s) or other Internet site(s)*

Nothing to Report

### *Technologies or techniques*

Nothing to Report

### *Inventions, patent applications, and/or licenses*

Nothing to Report

### *Other Products*

Nothing to Report

## **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

### **What individuals have worked on the project?**

Name: Vivek Krishna

Project role: Post-doctoral fellow

Nearest person month worked: 6

Contribution to project: Dr. Krishna has performed in vitro assays on freshly isolated intestinal smooth muscle cells and fibroblasts. Dr. Krishna has also performed in vivo experiments assessing the role of NR4A1 and its activation in experimental models of IBD.

Funding support:

Name: Laurie Alston

Project role: Laboratory technician

Nearest person month worked: 4

Contribution to project: Ms. Alston has support all in vivo work in for this contract. In this role, Ms. Alston isolated samples for experimental outcomes, process histological samples, images slides and performs biochemical assays to assess tissue inflammation in samples.

Funding support: Crohn's & Colitis Canada Operating Grant.

Name: Kyle Flannigan

Project role: Post-doctoral fellow

Nearest person month worked: 4

Contribution to project: Dr. Flannigan has performed in vivo experiments assessing the role of NR4A1 and its activation in experimental models of IBD. Dr. Flannigan has also helped to characterize the inflammatory cell infiltrate in experimental models of colitis and its regulation by NR4A1.

Funding support: Alberta Innovates Health Solutions Post-doctoral Fellowship

### **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

No changes have occurred during this reporting period.

### **What other organizations were involved as partners?**

No other organizations are involved as partners.

## **SPECIAL REPORTING REQUIREMENTS**

### **COLLABORATIVE AWARDS**

Not applicable

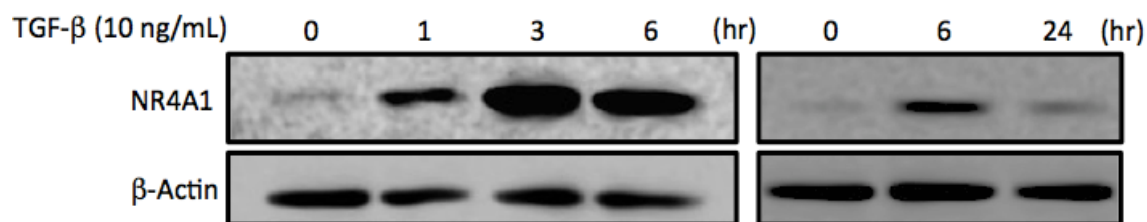
### **QUAD CHARTS**

Not applicable

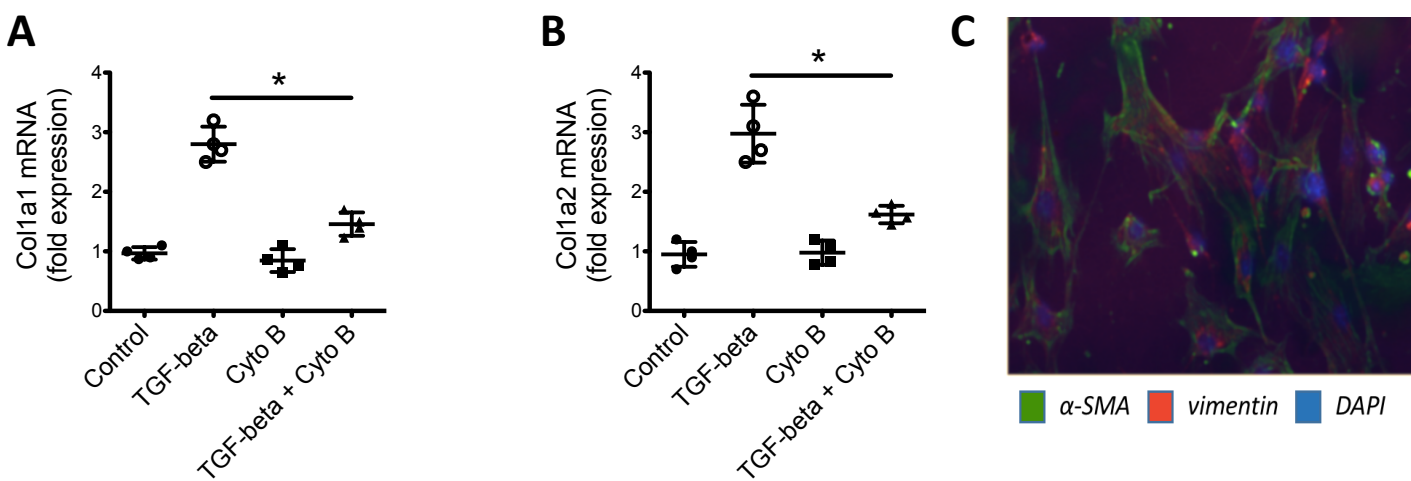
## **APPENDICES**

## **Figures**

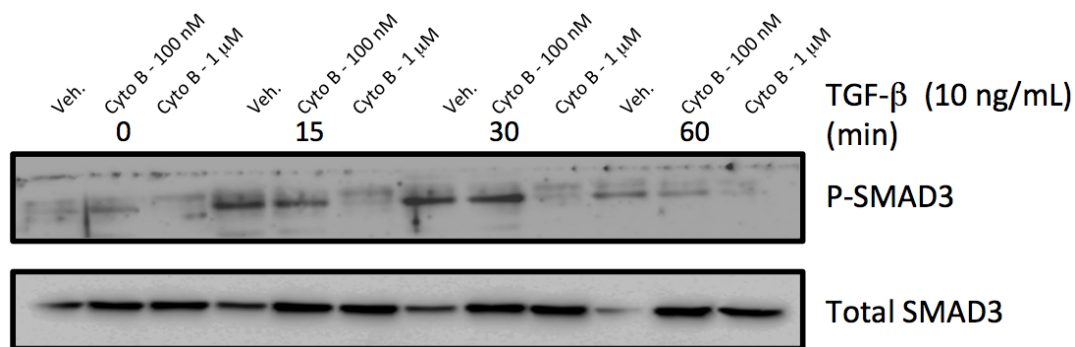




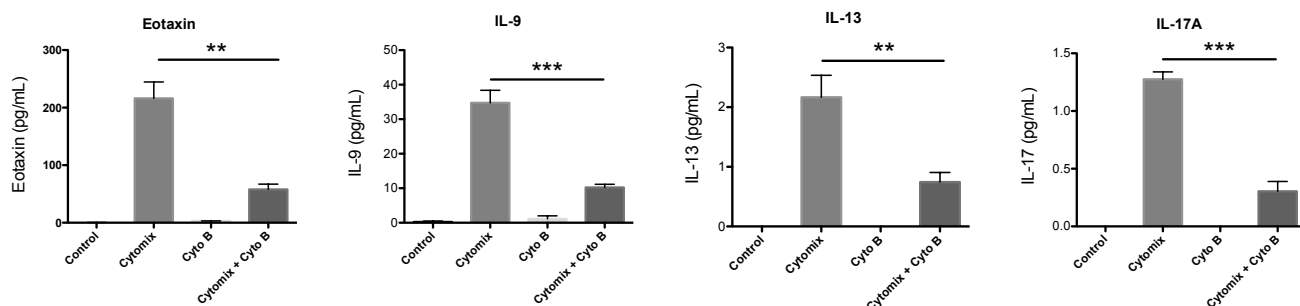
**Figure 1.** Short-term stimulation of primary human intestinal fibroblasts (0-6 hr) with TGF- $\beta$ 1 enhances NR4A1 expression, an effect that is reduced during long-term stimulation (24 hr)



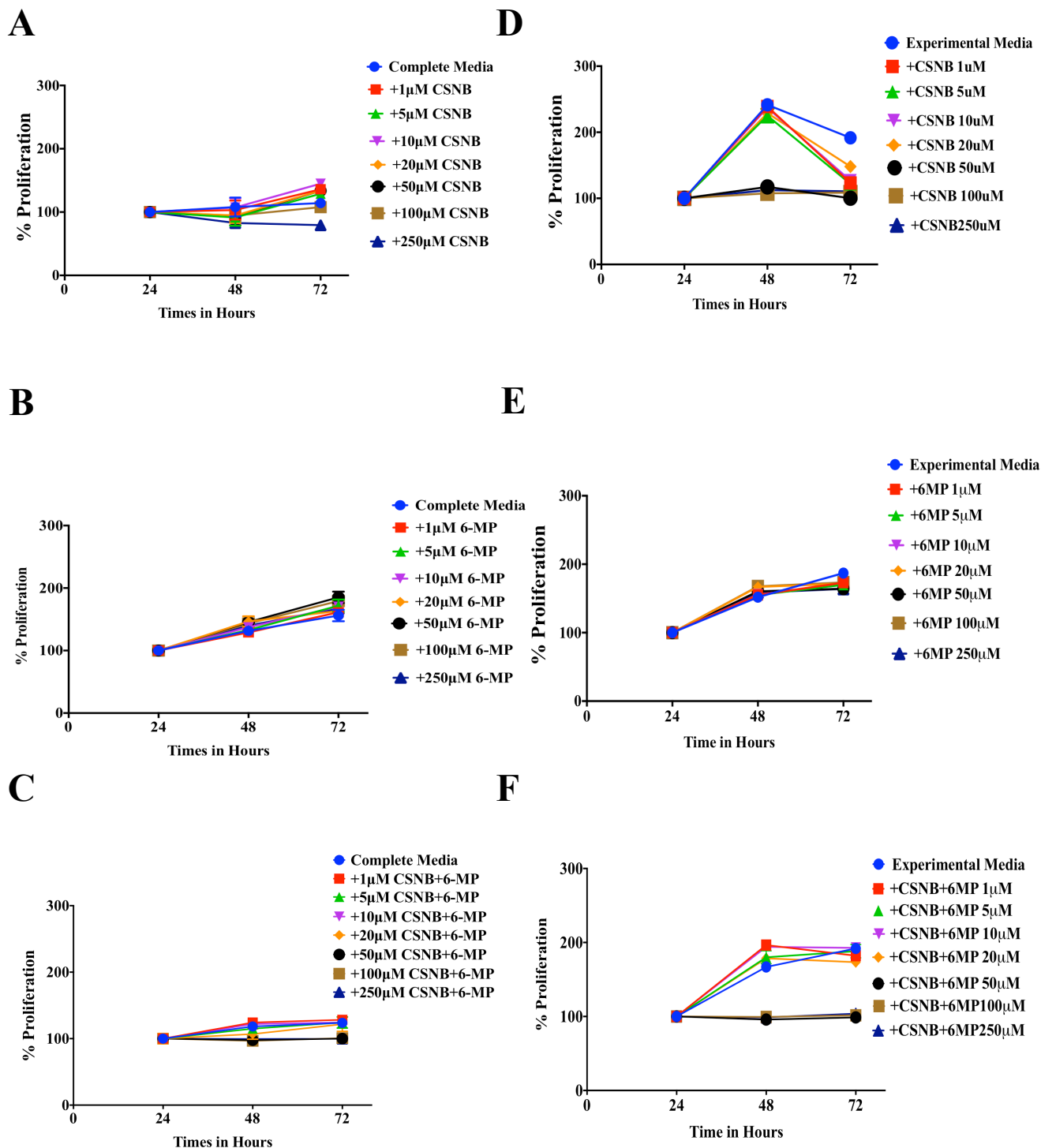
**Figure 2.** Selective activation of Nr4a1 with cytosporone B (Cyto B; 0.5  $\mu$ M) attenuates TGF- $\beta$ 1-induced expression of *Col1a1* (A) and *Col1a2* (B) from primary mouse intestinal myofibroblasts (C), characterized by their positive staining for vimentin and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA). TGF- $\beta$ 1 @ 10 ng/mL for 16 hr; n = 4; \* denotes p<0.05.



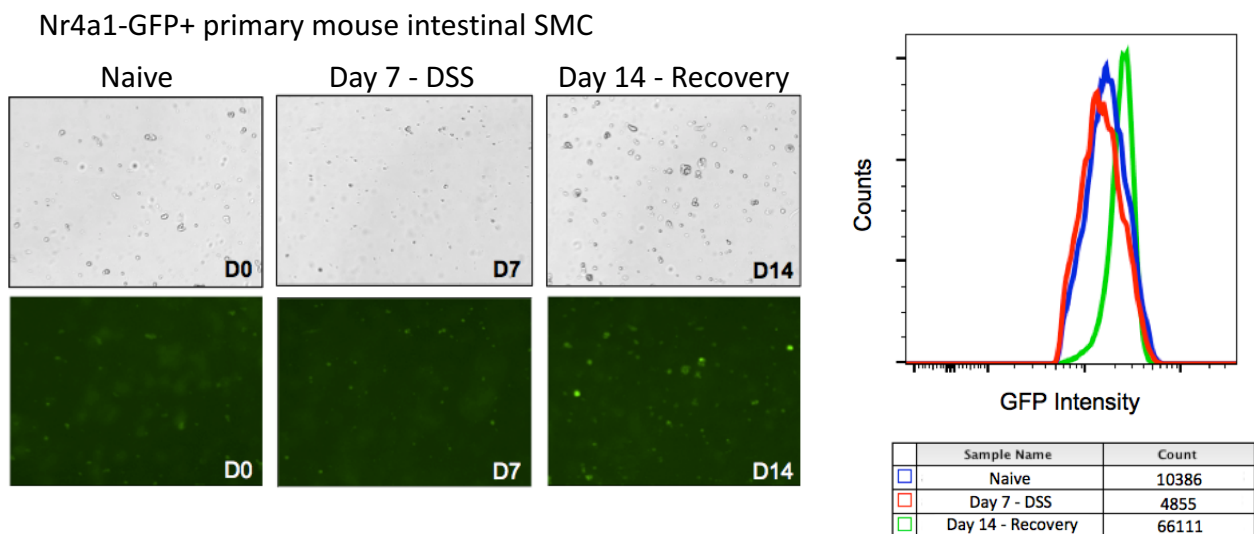
**Figure 3. B)** Selective activation of Nr4a1 with cytosporone B (Cyto B) attenuates TGF-beta1-induced SMAD3 phosphorylation in primary mouse ileal fibroblasts.



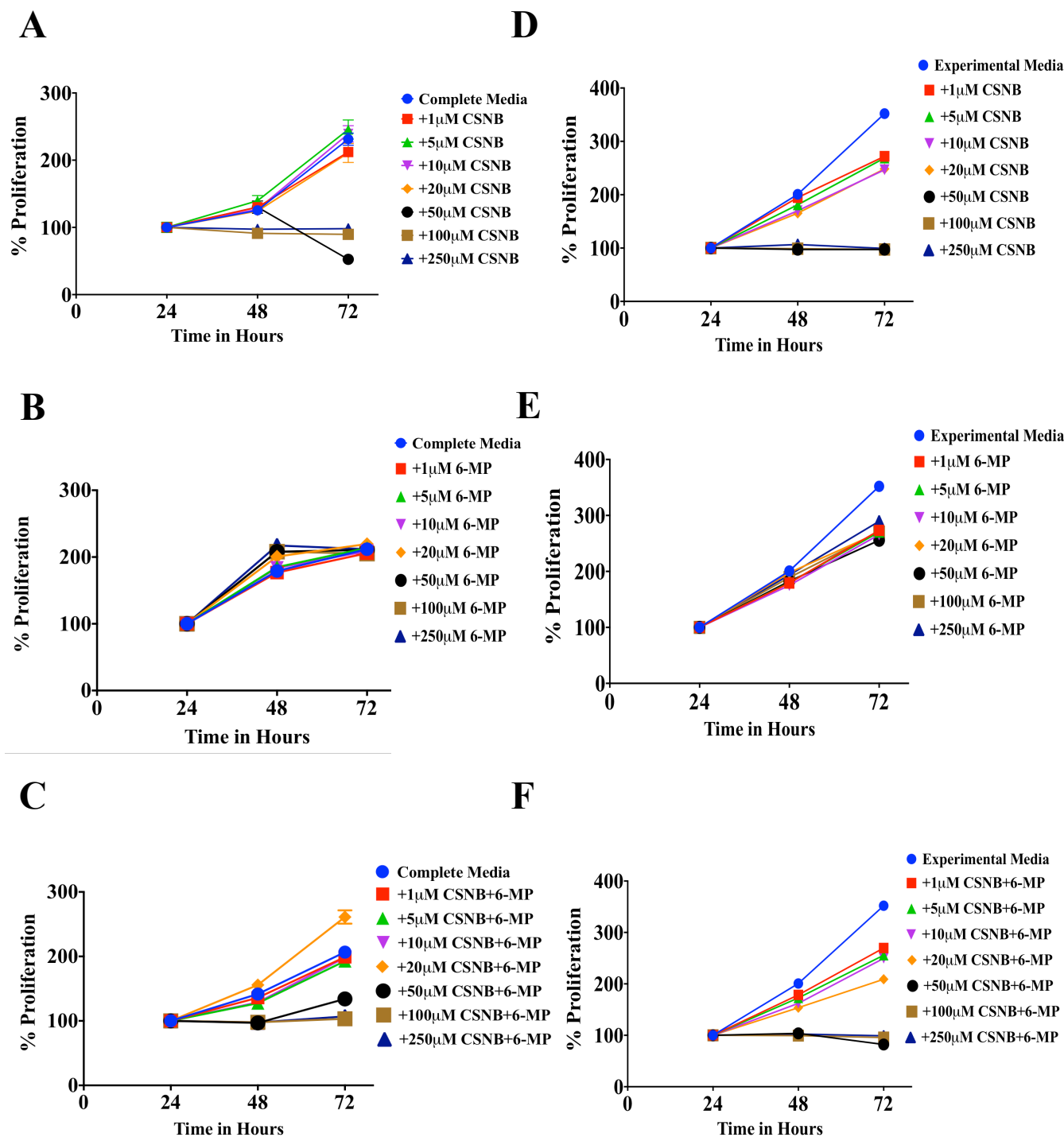
**Figure 4.** Selective activation of Nr4a1 with cytosporone B (Cyto B; 0.5  $\mu$ M) attenuates cytomix-induced inflammatory mediator release from primary mouse intestinal myofibroblasts. Cytomix (TNF- $\alpha$ ; IL-1 $\beta$ ; IFN $\gamma$  each @ 10 ng/mL) for 16 hr; n = 4; \*\* denotes p<0.01. \*\*\* denotes p<0.005.



**Figure 5.** Activation of NR4A1 with cytosporone B (CSN-B), but not 6-mercaptopurine (6-MP), attenuates serum-induced proliferation of primary intestinal human fibroblasts. Treatment of primary human intestinal fibroblasts with increasing concentration of CSN-B and 6-MP in serum-free complete (A-C) and serum containing experimental media (D-F). The cell proliferation was assessed by MTT assay over the course of 3 days. N = 3.

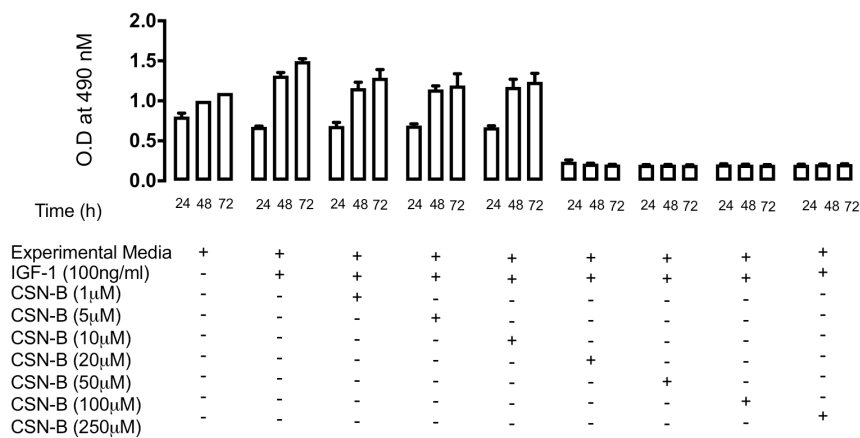


**Figure 6.** A) DSS-treated Nr4a1-GFP mice exhibit reduced Nr4a1 expression in freshly isolated intestinal smooth muscle cells at the peak of inflammation (Day 7), but increased expression during the recovery phase (Day 14).

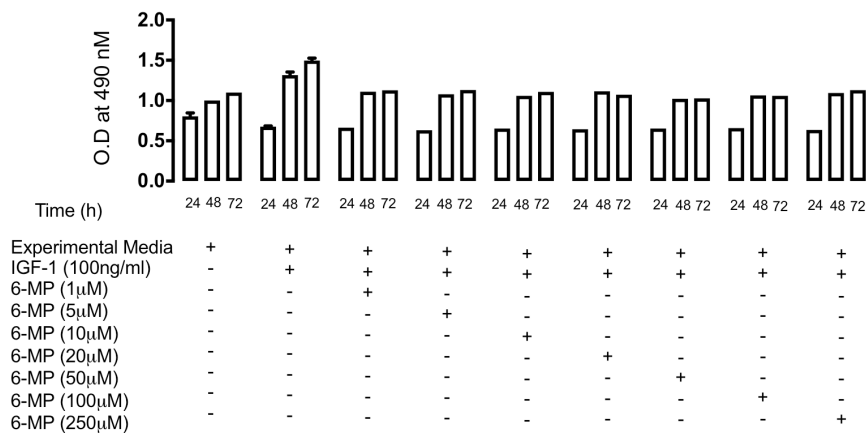


**Figure 7.** Activation of NR4A1 with cytosporone B (CSN-B), but not 6-mercaptopurine (6-MP), attenuates serum-induced proliferation of primary intestinal human smooth muscle cells. Treatment of primary human intestinal smooth muscle cells with increasing concentration of CSN-B and 6-MP in serum-free complete (A-C) and serum containing experimental media (D-F). The cell proliferation was assessed by MTT assay over the course of 3 days. N=10; data are expressed as mean±SD.

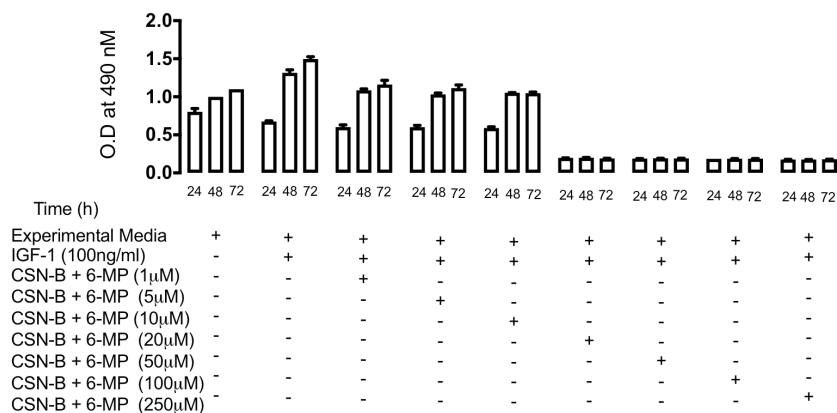
**A**



**B**

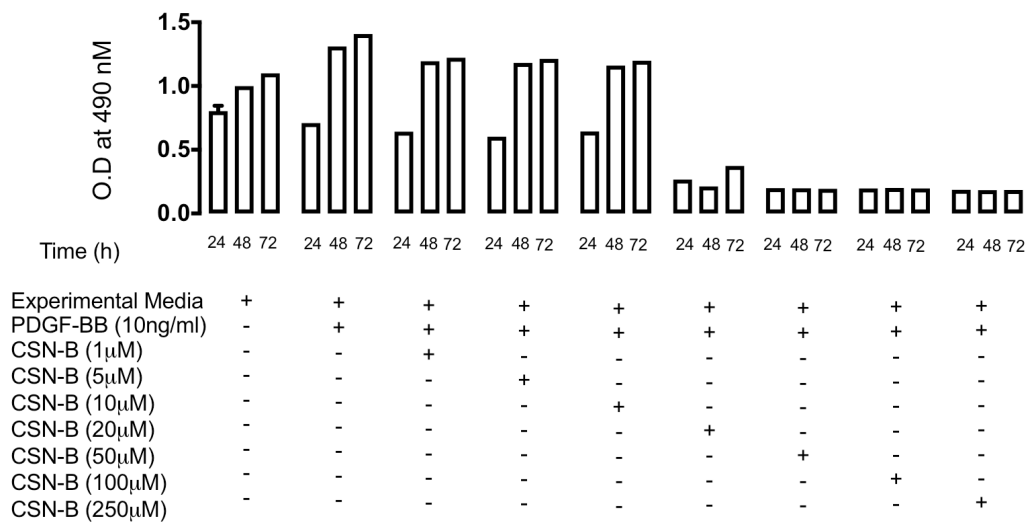


**C**

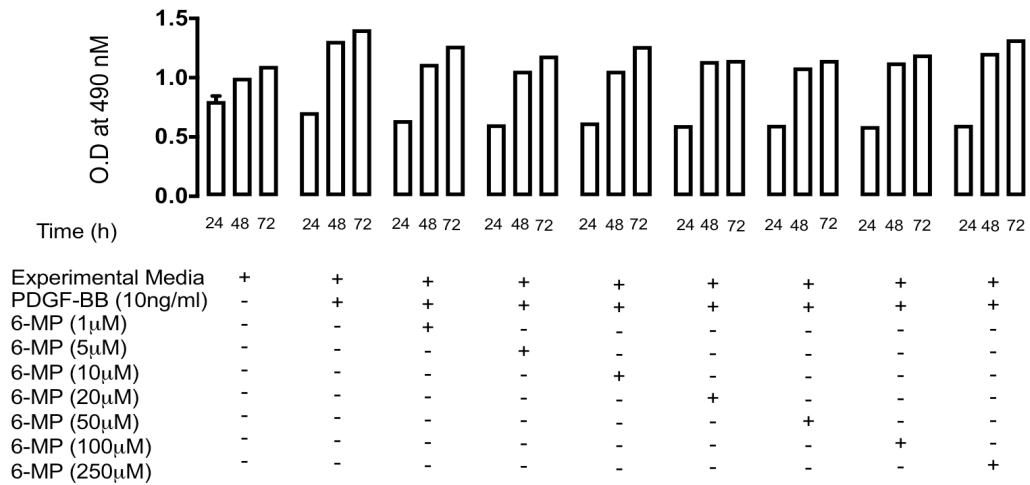


**Figure 8.** IGF-1-induced proliferation of primary human intestinal smooth muscle cells is attenuated by activation of NR4A1 by cytosporone B (CSN-B), but not 6-mercaptopurine (6-MP). Human intestinal smooth muscle cells were treated with increasing concentration of CSN-B and 6-MP and stimulated with IGF-1 (100 ng/mL). Cell proliferation was assessed for days 1-3. N=3; data are expressed as mean±SD.

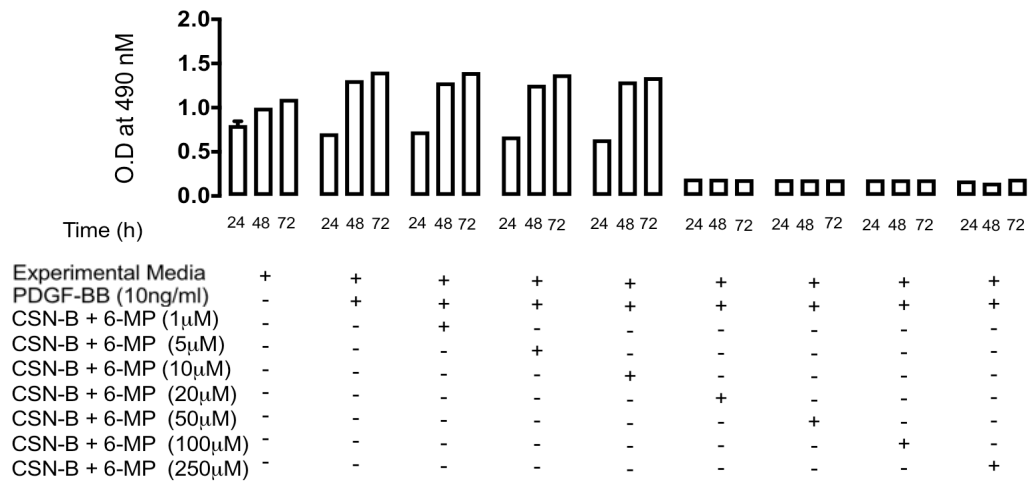
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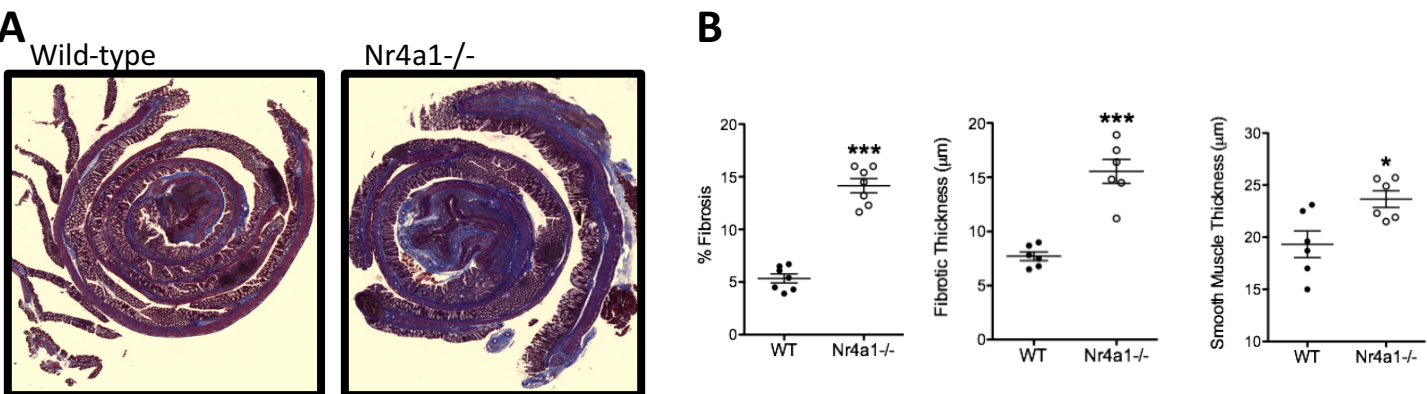
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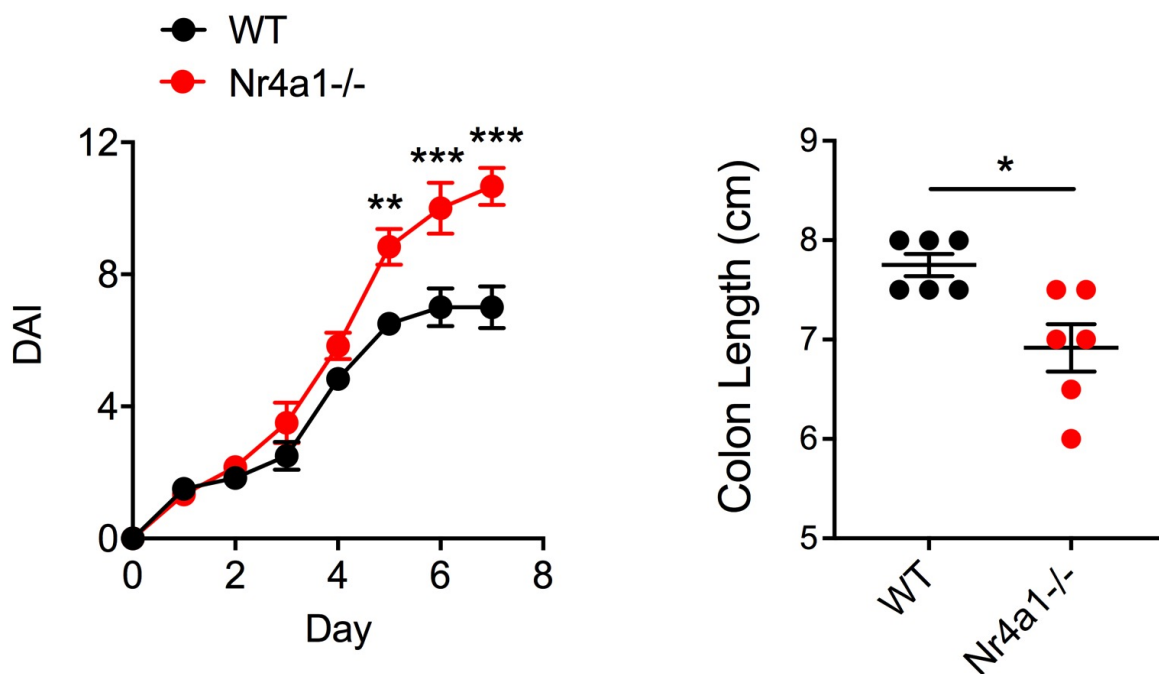
C



**Figure 9.** PDGF-BB-induced proliferation of primary human intestinal smooth muscle cells is attenuated by activation of NR4A1 by cytosporone B (CSN-B), but not 6-mercaptopurine (6-MP). Human intestinal smooth muscle cells were treated with increasing concentration of CSN-B and 6-MP and stimulated with PDGF-BB (100 ng/mL). Cell proliferation was assessed for days 1-3. N=3; data are expressed as mean±SD.

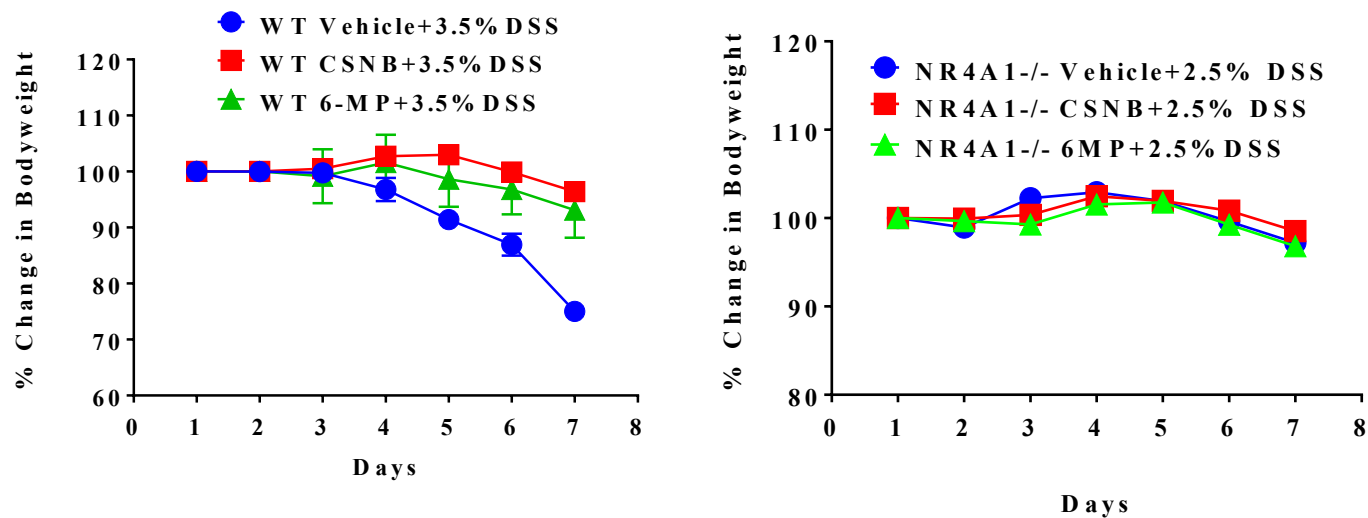


**Figure 10.** Nr4a1<sup>-/-</sup> mice exhibit enhanced intestinal fibrosis (evidenced by increased Masson's trichrome staining; A) and muscle thickening following the induction of chronic intestinal inflammation (dextran sulphate sodium – DSS: 2.5% for 7 days followed by 7 days of recovery; repeated 3 times). (B) Pooled histological analysis; n = 5-6; \* denotes p<0.04 compared to WT; \*\*\* denotes p<0.001 compared to WT.

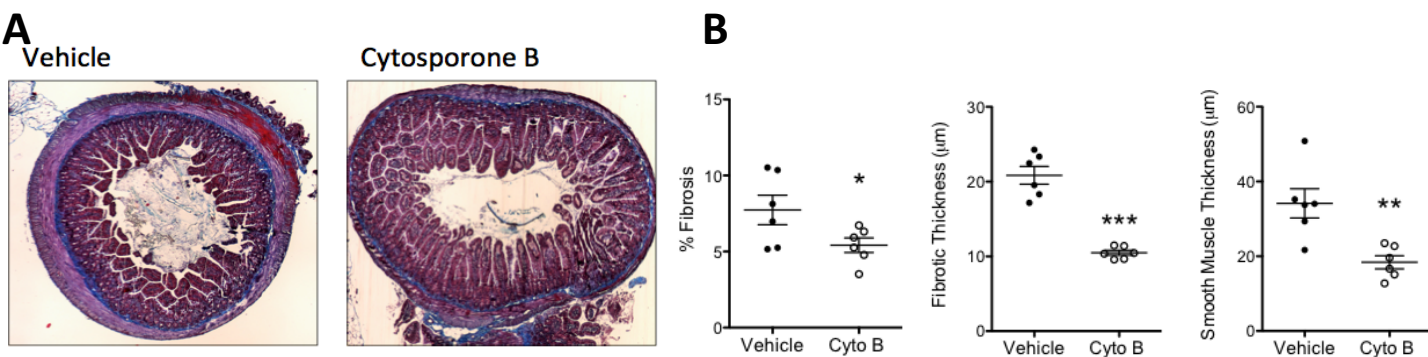


**Figure 11.** Nr4a1<sup>-/-</sup> mice exhibit enhanced susceptibility in acute experimental colitis as evidenced by significantly elevated disease-activity index (DAI) scores and reduced colon length. N = 6; \* denotes p<0.05; \*\* denotes p<0.005; \*\*\* denotes p<0.001.





**Figure 12.** Activation of Nr4a1 with daily administration of cytosporone B (CSN-B) or 6-mercaptopurine (6-MP) attenuates weight loss in acute experimental colitis in WT, but not Nr4a1-/- mice. N = 6. **NOTE:** to attempt to equalize the severity of disease between WT and Nr4a1-/- mice, WT mice were treated with 3.5% DSS and Nr4a1-/- treated with 2.5% DSS.



**Figure 13.** Selective activation of Nr4a1 with cytosporone B (Cyto B) attenuates fibrosis and smooth muscle thickening in the SAMP1/YitFcsJ model of spontaneous ileal CD-like disease, as evidenced by Masson Trichrome staining (A) and pooled histological analysis (B). NOTE: Cyto B was started at 10 weeks of age after the onset of ileal inflammation. n = 5-6; \* denotes  $p < 0.05$  compared to vehicle; \*\* denotes  $p < 0.005$  compared to vehicle; \*\*\* denotes  $p < 0.001$  compared to vehicle.